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Wolfram syndrome-associated mutations lead to instability and proteasomal degradation of wolframin

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Abstract **Wolfram syndrome is caused by mutations in *WFS1* encoding wolframin, a polytopic membrane protein of the endoplasmic reticulum. Here, we investigated the molecular pathomechanisms of four missense and two truncating mutations in *WFS1*. Expression in COS-7 cells as well as direct analysis of patient cells revealed that *WFS1* mutations lead to drastically reduced steady-state levels of wolframin. All mutations resulted in highly unstable proteins which were delivered to proteasomal degradation. No wolframin aggregates were found in patient cells suggesting that Wolfram syndrome is not a disease of protein aggregation. Rather, *WFS1* mutations cause loss-of-function by cellular depletion of wolframin.**

Keywords: Wolfram syndrome; *WFS1*; Wolframin; Mutation; Endoplasmic reticulum

1. Introduction

Wolfram syndrome is a rare autosomal-recessive disorder defined by the association of early-onset diabetes mellitus and optic atrophy. Additional neurological and endocrinological manifestations may be present [1]. The disease phenotype is caused by mutations in *WFS1* on 4p16 [2,3] encoding the 100 kDa protein wolframin. Secondary structure analysis predicted wolframin as a multispanning membrane protein with nine transmembrane segments [3]. Wolframin is a resident component of the endoplasmic reticulum (ER) [4] with an N_{cyt}/C_{lum} orientation in the ER membrane [5]. Recent studies suggested an involvement in the regulation of ER stress [6–8].

To date, a wide spectrum of different mutations in *WFS1* is known to cause Wolfram syndrome [9]. Most of them represent inactivating mutations implying that they cause a loss-of-function phenotype. Consistently, our previous study and that of others showed lack of wolframin in fibroblasts derived from patients with genotypes W371X/R629W, fs343X396/fs343X396 [5] or G695V/W648X [7]. However, there is no current concept about how wolframin is inactivated. We proposed that instability of *WFS1* transcripts is associated with truncating mutations W371X and F343fsX396, whereas mutation R629W causes instability of wolframin protein. Fonseca and co-authors [7] suggested that missense mutations G695V and P724L cause inactivation by formation of wolframin aggregates. Here, we investigated the effects of six different missense mutations and truncating mutations on the expression level, stability, degradation and the intracellular fate of wolframin. We show that all these *WFS1* mutations result in unstable proteins. Proteasomal degradation but not formation of aggregates contributes to cellular depletion of wolframin.

2. Materials and methods

2.1. Generation of WFS1 constructs

Wolframin mutants were generated by the megaprimer mutagenesis protocol. A mutated megaprimer was amplified using an outside wild-type (WT) primer (forward or reverse) and a middle mutagenic primer (forward or reverse). This megaprimer was used in a second round of PCR together with the appropriate outside primer. Mutants F883X and P885L were generated by a single PCR step. Human *WFS1* in pIRES2 [5] was used as template for PCR. Primers were: WT-F, 5′-atggactccaacactgctccg-3′; WT-R, 5′-tcaggccgccgacaggaatc-3′; P504L-R, 5′-catagagcaggcacaggacgctg-3′; R629W-F, 5′-ctgacgtggagctccatgg-3′; W700X-R, 5′-gaagcggccggtctacgtg-3′; P724L-F, 5′-catcaacatgctcctgttcttcatc-3′; F883X-R, 5′-tcagaaaaagaagtcgaagcc-3′; P885L-R, 5′-tcaggccgccgacaggaataggaagaa-3′. A high fidelity Taq polymerase (Roche) was used for all PCR amplifications. *WFS1* fragments were ligated into pIRES2 (Clontech) and sequenced using an automated ABI310 sequencer.

2.2. Cell culture and transfection

Fibroblasts derived from a healthy individual as well as from Wolfram syndrome patients carrying mutations W371X/R629W, fs343X396/fs343X396 [5] or W700X/W700X were used in this study. Fibroblasts and COS-7 cells were cultured at 37 °C in DMEM supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. COS-7 cells were transfected with *WFS1* constructs in 12-well tissue culture plates using Metafectene (Biontex). Twenty-four hours post-transfection, cells were either used for metabolic labelling experiments or harvested by trypsinisation. When indicated, cells were grown in the presence of lactacystin (Calbiochem) or E-64 (Sigma).

2.3. Pulse-chase experiments

Pulse-chase experiments were performed as described previously [5]. In brief, construct-transfected COS-7 cells were pulse-labeled for 30 min with 100 μCi/ml ^{35}S-labeled methionine/cysteine (NEN) and then chased for indicated time periods. At each time point, cells were lysed in IP buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol) containing 1% SDS and protease inhibitors (mini complete, Roche) for 30 min at 0 °C. Cleared extracts were subjected to immunoprecipitation with anti-WoN antibody bound to Protein A-sepharose and analysed by SDS/PAGE (3–8% NuPage Tris–acetate gels, Invitrogen) and autoradiography.

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2.4. Immunofluorescence

Fibroblasts were grown until sub-confluency and then fixed in ice-cold methanol (100%) for 20 min. Blocking was done in 1 × PBS containing milk powder (5%) followed by incubation with anti-WoN (1:100) in blocking solution for 90 min at room temperature. Incubation with secondary antibody was performed for 60 min using anti-rabbit IgG-FITC (1:360, Sigma). Slides were mounted in fluorescent mounting medium (DAKO), sealed and then analysed in a confocal microscope with the appropriate filter.

2.5. Detergent extraction

Transfected COS-7 cells (~1×10^5 cells) were lysed in 100 µl 1 × PBS pH 7.4, 10% glycerin, 2 mM EDTA, 1% Nonidet P-40 (Sigma) and protease inhibitors (mini complete, Roche) for 30 min on ice. Insoluble material was recovered by centrifugation at 20 000 × *g* for 15 min and solubilised in 50 µl 10 mM Tris/HCl pH 7.4, 1% SDS for 10 min at room temperature. After addition of 200 µl detergent buffer, samples were sonicated for 20 s. TCA-precipitated proteins derived from soluble and insoluble fractions were then separated on 7% Tris–acetate gels (Invitrogen) and analysed by immunoblotting.

2.6. RT-PCR analysis

RNA from cultured fibroblasts was extracted using TRIZOL (Invitrogen) and treated with DNAse (Promega) according to the manufactures recommendations. First-strand cDNA was synthesised from total RNA using oligo (d)T primers and Superscript II (Invitrogen). RT-PCR amplification was performed with human *WFS1*-specific primers (F: 5′-cctgccactgcgtctgaag-3′; R: 5′-ccacatccaggttgggctc-3′) and *GAPDH*-specific primers (F: 5′-gtccactggcgtcttcacca-3′; R: 5′-ggtggcagtgatggcatggac-3′) and analysed after different cycle numbers. The *WFS1* primers used here amplify a region of the gene which spans an intron; the *GAPDH* primers correspond to published primers for pseudogene-free amplification of the *GAPDH* gene [10].

3. Results

3.1. WFS1 mutations

In this study, we investigated four missense and two truncating *WFS1* mutations. The missense mutations correspond to sequence portions encoding transmembrane helical (P504L), cytoplasmic loop (R629W) and lumenal C-terminal (P724L, P885L) domains of wolframin. Of these, P504L, R629W and P885L belong to the known rare homozygous missense mutations [2,11,12]. The W700X mutation represents a novel inactivating mutation present in homozygosity in a female patient. It leads to truncation of a major portion of the hydrophilic C-terminus. The F883X mutation causes deletion of the last eight amino acid residues and mimics a group of frameshift mutations affecting the very C-terminus of wolframin [2,11,13].

3.2. WFS1 mutations lead to cellular depletion of wolframin

To assess steady-state concentrations of wolframin mutants, we used transient expression of wild-type and mutated constructs in COS-7 cells. Cells expressing the wild-type wolframin showed very strong signals at about 100 kDa (Fig. 1A). In contrast, the steady-state concentrations of mutants were significantly reduced when compared to the wild-type protein. The direct analysis of endogenous mutated wolframin in patient cells revealed a more drastic reduction (Fig. 1B): signals corresponding to $\text{wolframin}_{\text{W700X}}$ were almost undetectable in respective patient cells and $\text{wolframin}_{\text{R629W}}$ was markedly reduced compared to control cells. We conclude that *WFS1* mutations, both truncating and missense mutations, cause cellular depletion of wolframin.

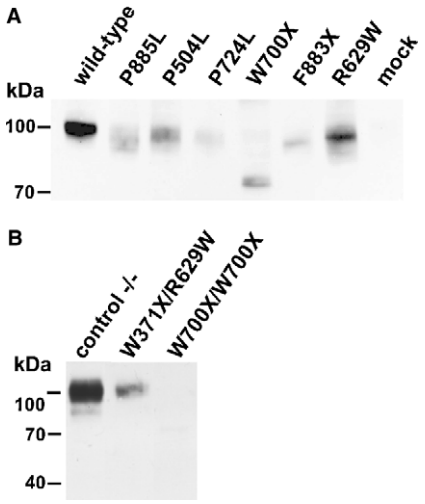

Fig. 1. Steady-state levels of mutated and truncated forms of wolframin. (A) Construct-transfected COS-7 cells were lysed in SDS buffer and analysed by immunoblotting with anti Wo-N. (B) Immunoblot analysis of control fibroblasts and of two patients (W371X/R629W, W700X/W700X). Aliquots (5×10^4 cells) were resolved by SDS/PAGE and blotted with anti-WoN and anti-β actin.

3.3. Truncating mutations of WFS1 are associated with stable transcripts

Nonsense and frameshift mutations generate premature stop codons which may cause mRNA degradation through nonsense-mediated mRNA decay (NMD) and thus, absence of the encoded protein [14]. We asked whether late truncating mutations W700X and F883X cause instability of *WFS1* transcripts. Since reliable RT-PCR analysis of construct-transfected cells is hampered by residual plasmid DNA, we directly determined levels of *WFS1* transcripts in W700X/W700X fibroblasts. For comparison, we re-analysed patient fibroblasts with F343fsX396 present in homozygosity [5]. RT-PCR analysis revealed stable *WFS1* transcripts in W700X/W700X cells (Fig. 2). Surprisingly, we also obtained stable *WFS1* transcripts in F343fsX396/F343fsX396 fibroblasts what was in contrast to our previous findings [5]. False-positive results due to amplification from residual genomic DNA could be excluded. We speculate that the interference of pseudogene sequences by the use of different *GAPDH* primers in the former study has

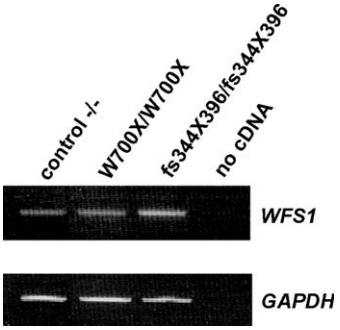

Fig. 2. Semi-quantitative RT-PCR analysis of *WFS1*. First-strand cDNAs derived from two patients (W700X/W700X, fs343X396/fs343X396) and a control were analysed by PCR using *WFS1*- or *GAPDH*-specific primers as a control. PCR products corresponding to 26 cycles are shown.

made normalisation against *GAPDH* mRNA erroneous. We conclude that *WFS1* mutations carrying premature stops downstream of F343fsX396 encode stable transcripts.

3.4. Missense and truncating mutations in WFS1 reduce the stability of wolframin

The *WFS1* mutations investigated here may result in unstable proteins as shown previously for the R629W mutation [5]. To determine possible differences in protein stability between wild-type and mutant wolframin, we performed pulse-chase experiments using construct-transfected COS-7 cells. As shown in Fig. 3, wild-type wolframin was stable over a chase period of 6 h and reduced to about 50% after 24 h. In contrast, the amounts of wolframin mutants decreased more rapidly. Mutants P504L and P724L were decreased to about 50% after a chase period of 6 h whereas mutants R629W, P885L, W700X and F883X were almost reduced to background levels after this chase period. This demonstrates that *WFS1* mutations, at least those investigated here, result in unstable wolframin proteins which exhibit markedly reduced half-lives.

3.5. Wolframin mutants are degraded by the proteasome

Many mutated proteins are delivered to the proteasomal pathway for rapid degradation. We asked whether proteasomal degradation is responsible for low concentrations of wolframin mutants. Therefore, the effect of the proteasome inhibitor lactacystin on steady-state levels of wolframin was assessed. Levels of wild-type wolframin were only slightly increased whereas a marked accumulation of the mutants P504L, R629W, P724L and P885L was observed in the presence of the inhibitor (Fig. 4A). The increase of mutants W700X and F883X was less pronounced although significant. These data indicate that degradation of wolframin mutants transiently expressed in COS-7 is mediated by the proteasome pathway.

To analyse degradation mechanisms of endogeneous mutants, fibroblasts from two patients (W371X/R629W, W700X/W700X) and a control were treated with either lactacystin or E-64, an inhibitor of cysteine proteases. In the presence of lactacystin, we observed a marked increase of wolframin$_{R629W}$ (Fig. 4B). Wolframin$_{W371X}$ and wolframin$_{W700X}$ which were almost undetectable in untreated fibroblasts, accumulated to significant levels. The inhibitor E-64 had almost no effect on wolframin levels in patient cells (Fig. 4B). We conclude that the cellular depletion of wolframin in patient cells is essentially caused by proteasomal degradation.

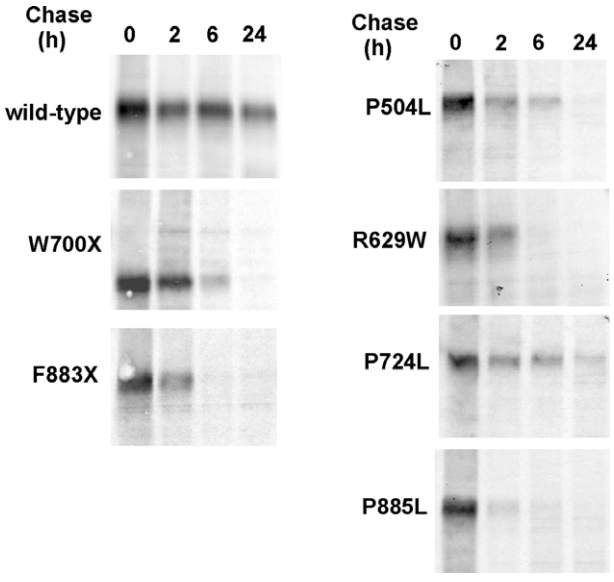

Fig. 3. Stability of wild-type and mutant wolframin. Construct-transfected COS-7 cells were radiolabeled with ^{35}S-cysteine/methionine and analysed after the indicated time periods (chase) by immunoprecipitation followed by SDS/PAGE and autoradiography.

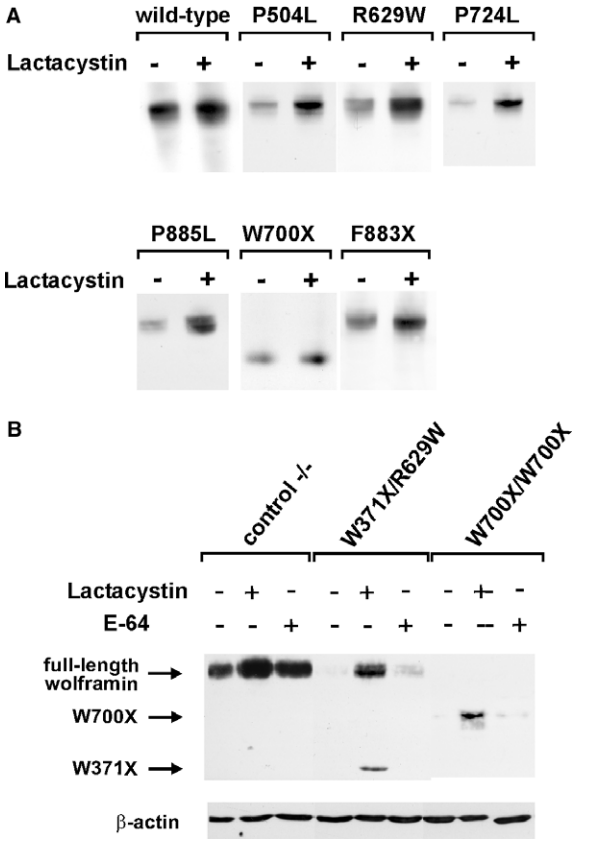

Fig. 4. Degradation of wolframin by the proteasome. (A) Construct-transfected COS-7 cells were treated with lactacystin (50 μM) or solvent (DMSO) only for 16 h. Cells were harvested and analysed by immunoblotting. (B) Fibroblasts derived from two patients (W371X/R629W, W700X/W700X) and a control were treated with either lactacystin (50 μM) or E-64 (10 μM) for 2 days and then analysed by immunoblotting.

3.6. Aggregate formation does not contribute to loss-of-function of wolframin

A recent study suggested that mutation P724L leads to the formation of detergent-insoluble aggregates of wolframin when expressed in COS-7 cells [7]. We asked whether also other *WFS1* mutations induce aggregation of wolframin. Such aggregates may escape detection by direct analysis of cell lysates. To investigate possible differences in detergent extractability of wild-type versus mutant wolframin, construct-transfected COS-7 cells were solubilised and then separated by centrifugation into detergent-soluble (supernatant) and detergent-insoluble (pellet) fractions. Whereas wild-type wolframin was found exclusively in the supernatant fraction, mutations R629W, W700X and F883X significantly increased the levels of insoluble forms of the protein (Fig. 5A) suggesting conversion into

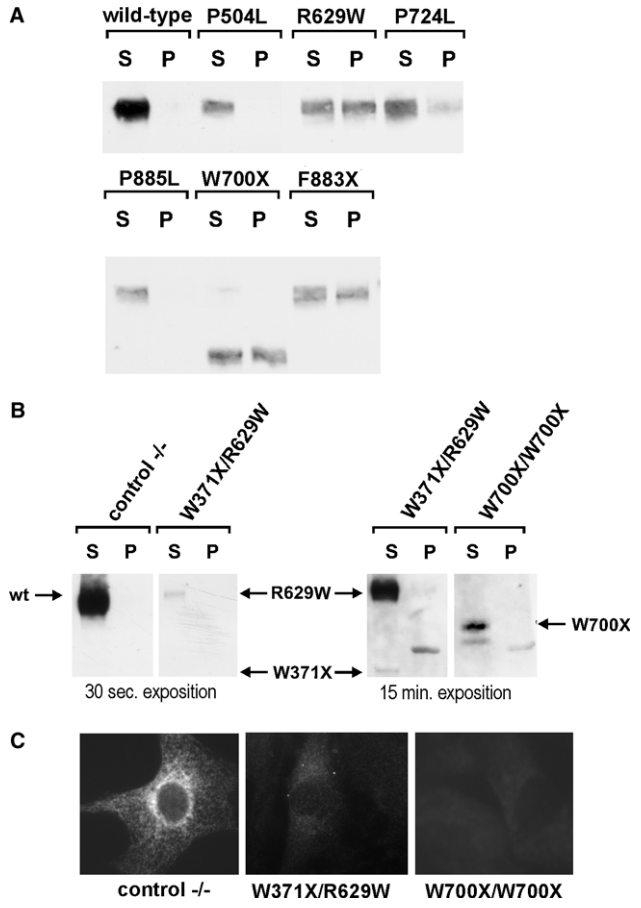

Fig. 5. The fate of mutated wolframin. Either (A) construct-transfected COS-7 cells or (B) patient fibroblasts were fractionated into detergent-insoluble pellet (P) and detergent-soluble supernatant (S) fractions and analysed by immunoblotting. A longer exposition time was required to visualise endogeneous mutants. (C) Immunofluoresence analysis of wolframin of control and patient fibroblasts. A reticular pattern of staining was found with control fibroblasts whereas patient fibroblasts showed very weak staining.

aggregates. Mutants P724L, P504L and P885L were primarily detected in the detergent-soluble fraction.

It cannot be excluded that the aggregate formation is related to the system of transient expression in COS-7 cells. We, therefore, investigated the fate of endogeneous wolframin mutants in patient cells. As shown in Fig. 5B, wolframin$_{R629W}$ and wolframin$_{W700X}$ were entirely released into the supernatant fraction similar to wild-type wolframin. Immunofluorescence studies confirmed that formation of aggregates is not involved in loss-of-function of wolframin in patients (Fig. 5C). Whereas control cells showed a strong and reticular pattern of staining with anti-WoN, only background staining was found with patients cells with no signs of punctuate-like staining. We conclude that conversion of mutant forms of wolframin to insoluble aggregates is not the leading pathomechanism in patient cells. Rather, mutated proteins appear to be rapidly degraded at an early stage of biogenesis.

4. Discussion

We show that mutations causing Wolfram syndrome are associated with cellular depletion of wolframin irrespective of whether the mutations encode full-length or truncated proteins. Transient expression of constructs carrying missense mutations or C-terminal deletions revealed reduced steady-state levels compared to wild-type wolframin. In agreement with this finding, wolframin is also absent in patient cells: in a newly identified patient with mutation W700X present in homozygosity, in a patient carrying W371X/R629W [5] and a further patient with mutations G695S/W648X [7] confirming that depletion of wolframin is underlying the disease.

All mutations investigated here obviously encode stable transcripts. We demonstrated stable W700X transcripts in a patient indicating that a premature stop at this position does not trigger NMD. Most likely, this is also true for downstream mutations like F883X. Re-investigation of a mutation generating a premature stop upstream of W700X, F343fsX396, also revealed stable *WFS1* transcripts. This is in line with the rule that a premature stop must lie 50–55 nucleotides upstream of the last exon–exon junction to trigger NMD [15]. According to this rule, only mutations generating premature stops upstream of position 269 within wolframin should cause NMD. This applies to a minority of known *WFS1* mutations.

Pulse-chase experiments indicated that reduced half lives of wolframin mutants were responsible for low protein levels suggesting an increased degradation of wolframin mutants. Indeed, in the presence of lactacystin, transiently expressed wolframin mutants were stabilised. Similarly, treatment of W371X/R629W patient cells with lactacystin led to accumulation of wolframin$_{R629W}$ to almost wild-type levels. Thus, similar to other mutated proteins, wolframin is target of ER quality control [16]. Notably, most of the mutants were not restored to wild-type levels in the presence of lactacystin. It cannot be excluded that non-proteasomal mechanisms contribute to degradation of wolframin mutants. Furthermore, proteolytic fragments generated by N-terminal cleavage may escape detection by antibody anti-WoN.

In an increasing number of neurodegenerative disorders the pathophysiology is directly related to the deposition of aggregates of mutated proteins [17,18]. Indeed, when transiently expressed, mutants R629W, W700X and F883X partially formed insoluble aggregates. Thus, the probability of aggregate formation appears to be directly related to distinct amino acid substitutions or to the deletion of C-terminal amino acids causing changes in properties such as charges, secondary structure propensities or hydrophobicity. Our finding that transiently expressed P724L is nearly completely soluble is different from the study by Fonseca et al. [7] indicating that other influences like conditions of overexpression may additionally affect the fate of wolframin mutants. Nevertheless, our data provide evidence that formation of wolframin aggregates is not of major relevance in patient cells. We found endogeneous wolframin mutants exclusively in the detergent-soluble fraction. Indirect immunofluorescence studies supported these findings.

Our findings suggest that Wolfram syndrome is not a disease of protein aggregation. It is unlikely that the mutant proteins themselves contribute considerably to the neuronal degeneration in patients through a toxic gain of function. Rather, *WFS1* mutations lead to loss of normal cellular function by rapid degradation and cellular depletion of wolframin.

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